STUDIES ON FERRITIN

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STUDIES ON FERRITIN

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The fundamental role of ferritin as a storage form of iron has long been recognized. A structure, consisting of 20 identical subunits of apoferritin, the protein moiety, arranged in a regular manner around an iron micelle was supported by physical and chemical studies. Evidence showing the existance of isoferritins however; cast doubt on the 20 subunit model.

In an effort to determine the actual number of subunits and to ascertain their equivalence, structural studies on apoferritin were undertaken. Cleavage of apoferritin with cyanogen bromide (CNBr) was chosen as the first step because of the small number of methionine residues found on amino acid analysis. Based on the assumption of four methionine residues per 23,000 Dalton subunit it was expected that five peptides would result after CNBr cleavage. This expectation was not realized. Only four peptides were found in the CNBr digest, three of which have glutamic acid, glycine and isoleucine as N-terminal amino acids respectively. One of the peptides has been isolated and its N-terminal amino acid determined. The data indicates the presence of only three methionine residues per apoferritin subunit and therefore casts doubt on the previously determined number of each amino acid in the subunit and thus its actual molecular weight.

The molecular weight of dissociated apoferritin was reassessed

by polyacrylamide gel electrophoresis in the presence of sodium dodecyl sulfate and was determined to be 18,700 ± 600.

The data indicate that the subunit molecular weight is lower than originally thought and that apoferritin probably consists of 24 subunits.

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PART I

INTRODUCTION

The importance of iron as one of the most indespensible trace elements required by higher animals derives from the simple fact that due to its role in cellular respiration and in the transport of oxygen, it is required to sustain life. Approximately 70% of body iron is incorporated either into porphyrins where it forms an essential part of hemoglobin or into myoglobin and certain respiratory enzymes. The remaining 25-30% is bound to proteins designed for its storage or transport. These forms provide the organism with an internal reserve to protect against sudden losses due to bleeding or the consequences of low dietary intake. In addition they mediate to some degree the relatively coarse control by which iron absorption is regulated and the lack of an efficient excretory mechanism.

In 1894 Schmiedeberg recognized the existance of a storage form of iron which he called Ferratin. Laufberger (1) first isolated pure ferritin in 1937 and provided the impetus for the subsequent work of Granick and Michaelis (2)(3)(4) who showed that ferritin, isolated from horse spleen, consisted of a high molecular weight protein shell surrounding a central core of iron and phosphorous in the form of a ferric hydroxyphosphate micelle, $(FeOOH)_8(FeO: OPO_3H_2)$, from which it derived its characteristic red color. Furthermore they demonstrated that ferritin indeed served as a storage form whose iron could be mobilized for hemoglobin production. The complex which has been found in a wide

variety of species is water soluble and relatively stable to heat, properties which are exploited in its isolation from a variety of tissues. The protein shell which can be prepared by reduction, chelation and removal of the iron by dialysis was called apoferritin.

Apoferritin isolated from horse spleen has been the most widely studied and Rothen (5) first determined its molecular weight by analyzing its sedimentation and diffusion rates, arriving at a value of 465,000. Other published values ranged from 430,000 to 480,000 (6)(7). Evidence from x-ray diffraction studies confirmed a subunit structure as would be expected of a molecule with such a high molecular weight (8). The molecule was found to be stable in 10 M urea at neutral pH (9) and in organic solvents (10) but could be dissociated into subunits, sedimenting at 2-3 S, by incubation in 67 % acetic acid (11), 1-2 % sodium dodecyl sulfate (12) or 5-6 M guanidinium chloride (13). When the dissociating agents were removed, reaggregation of the subunits occurred.

Suran and Tarver (14) noted that apoferritin preparations contained small amounts of a faster sedimenting component which they identified as a dimer of the apoferritin molecule. Trimers and higher oligomers could been seen in polyacrylamide gel after electrophoresis or could be separated by fractionation on DEAE-cellulose columns. They could detect no appreciable differences in the amino acid composition or in fingerprints of tryptic digests of these heterogeneous fractions.

X-ray diffraction studies (8)(15) showed the apoferritin monomer to be a hollow, nearly spherical shell with inner and outer radii of

about 37 Å and 67 Å respectively. The best estimates of the number of subunits and their molecular weight, based on x-ray crystallographic (15) and chemical studies (16)(17)(19) led to the conclusion that apoferritin consisted of 20 identical subunits whose molecular weights ranged from 23,000 to 27,000, and which were situated at the vertices of a pentagonal dodecahedron (6).

The N-terminal pentapeptide from the tryptic digestion of apoferritin was isolated and its sequence determined by Suran (18). N-acetyl serine was found to be N-terminal. Mainwaring and Hofmann (19) determined the sequence of the C-terminal octapeptide and showed arginine to be the terminal amino acid. Their data supported the earlier findings of a structure with 20 subunits.

Several amino acid analyses of horse spleen apoferritin were published (10)(14)(16) and Table I shows the number of residues based on an assumed subunit molecular weight of 23,000.

Much less was known about the way in which apoferritin accumulates its iron, although ferritin synthesis was extensively studied in an effort to establish the controlling mechanism. A variety of iron compounds administered in vivo and in vitro were found to increase the ferritin content of different organs (20)(21)(22). This could be explained either as a net effect resulting from diminished turnover or as a de novo induction of apoferritin synthesis. The first case was based on the postulates of Drysdale and Munro (22) and Granick (23), that iron combined with existing apoferritin and stabilized the molecule

TABLE 1

AMINO ACID COMPOSITION OF HORSE SPLEEN APOFERRITIN
RESIDUES/23,000 DALTON SUBUNIT

Amino Acid	Ref (a)	Ref (b)	Best Integer
Cys	2.30	3.45	3
Asp	21.03	21.05	21
Thr	6.70	6.67	7
Ser	11.55	10.91	11
Glu	29.19	29.01	29
Pro	2.51	3.43	3
Gly	11.92	12.01	12
Ala	17.26	16.98	17
Val	8.59	8.59	9
Met	3.59	3.48	4
Ilu	4.56	4.25	5
Leu	30.52	30.35	30
Tyr	6.33	6.07	6
Phe	8.87	8.92	9
His	7.14	7.03	7
Lys	10.90	10.63	10
Arg	11.98	11.49	12

(a) Williams, MA & PM Harrison. Biochem. J. 110:265-280 (1968).

(b) Crichton, RR. Biochim. Biophys. Acta 194:34-42 (1969).

to proteolysis. The case for induction was originally proposed by Fineberg and Greenberg (20), and was supported by studies on amino acid incorporation.

Whatever the mechanism, it was clear that the biosynthetic response to iron required a means by which the element was sequestered in accessible form. Saltman and his colleagues (24), having demonstrated the tendancy of iron chelates to spontaneously polymerize into micelles proposed that apoferritin subunits assembled around a performed iron micelle in an all or none process. The most serious objection to this hypothesis was that previous in vivo experiments demonstrated that apoferritin itself was the first product of iron induction (20)(22). A second mechanism proposed that iron accumulation was a gradual process involving oxidation of ferrous iron and depended on the oxidative metabolism of the cell. In vitro experiments (25) and evidence from x-ray diffraction studies (26) gave circumstantial support to this hypothesis.

Thus at the time of the present study apoferritin could be described with some oversimplification, as a self-assembling, multi-subunit shell providing a cover for its iron core, access to which was provided by inter-subunit channels. One of the problems with the 20 subunit model was that all of the subunits could not occupy equivalent positions in the structure. Compatability with this model could occur however, if more than one kind of subunit were present. Support for this possibility came from studies of Richter (27)(28) who showed

differences in the electrophoretic mobilities of ferritins isolated from neoplastic and normal tissues. Other workers reported isoferritins for different organs of several species of normal animals (29)(30)(31).

The previous evidence based on the analyses of tryptic digests of whole apoferritin allowed no reliable conclusions to be drawn about the number of subunits in the molecule since the total number of peptides remained uncertain. Furthermore, studies on the amino acid composition of apoferritin could hardly be expected to reveal minor differences in primary structure. Although the evidence from chemical studies favored an identical subunit model, a nagging doubt existed.

The best approach to resolving this problem seemed to be through an expansion of the structural studies begun by Suran (18) and continued by Mainwaring and Hofmann (19). The large number of peptides found in tryptic digests of apoferritin and the posssibility that digestion itself might be incomplete argued against a purely enzymatic attack. Gross and Witkop (32) had described a specific chemical method using cyanogen bromide which cleaved peptide bonds at methionine residues and gave yields of greater than 90 %. Application of their cyanogen bromide method had been used successfully as a first step in the structural elucidation of proteins with a wide range of molecular weights (33)(34)(35)(36) and the small number of methionine residues established from amino acid analyses of apoferritin argued well for its application in this case. It was hoped that this technique would provide managable peptides from which further attacks on the primary structure could be

made either through enzymatic or other specific chemical techniques. Thus, as a result of cyanogen bromide cleavage of the apoferritin subunit one would expect five peptides, four with C-terminal homoserine derived from methionine and one with C-terminal arginine, the original C-terminal amino acid of apoferritin.

In fact, this expectation was not realized and the following describes the results of this study which although circumventing its original purpose led to a reevaluation of the subunit molecular weight.

PART II

EXPERIMENTAL PROCEDURE

1. Materials:

The ferritin used in this study was either prepared from horse spleen by a modification of the method of Granick (2), in that preparation was made in the cold (14), or was purchased from Pentex Biochemicals, Kankakee, IL (LOT 3-1, 2x crystallized, 102 mg/ml).

Blue Dextran 2000 and Sephadex for gel filtration was obtained from Pharmacia Fine Chemicals, Piscataway, N.J. Grades G-25, G-25 fine, G-50 fine, G-75 and G-75 superfine were used at various stages of this investigation.

Acrylamide, N, N^1-methylene bisacrylamide, N, N, N, N^1- tetramethyl-1,2 diaminomethane and 2-mercaptoethanol were purchased from Eastman Organic Chemicals Divisions, Rochester, N.Y.

Ponceau-S was obtained from Allied Chemical Corp. New York City, N.Y.

Coomassie Brilliant Blue R-250 was obtained from Mann Research Laboratories, Organgeburg, N.Y.

Dansyl chloride (5- dimethylaminoapthalene-1-sulfonyl chloride), dansyl derivatives of amino acids (Dansyl AMAC Kit), amino acid calibration standards, ninhydrin, 8-hydroxyquinoline, N-bromosuccinimide, tert-butyl hypochlorite, methyl cellosolve, hydrindantin and sodium dodecyl sulfate (SDS) were purchased from Pierce Chemical Co., Rockford, IL.

Polyamide-6 sheets, 20 x 20 cm, and cellulose MN-300 (Macherey-Nagel & Co.) were obtained from Brinkman Instruments Inc., Westbury, N.Y.

Carboxypeptidase A (bovine pancreas), carbonic anhydrase (bovine blood), ribonuclease (bovine pancreas) and trypsin (bovine pancreas) were products of Worthington Biochemical Corp., Freehold, N.J. Myoglobin (horse heart) was obtained from Calbiochem, Los Angeles, CA.

Other standard chemicals were reagent grade. Organic solvents were redistilled prior to use. Glass distilled, constant boiling HCl, 6.7 N, was used for hydrolysis.

2. Methods:

a. Preparation of Apoferritin

Apoferritin was prepared by a modification of the method of Granick and Michaelis (3) in that chromatography on a 25 x 2.5 cm column of Sephadex G-25 in 0.05 M acetate buffer, pH 4.8, was substituted for the dialysis step in their procedure. Apoferritin eluted in the void volume, well separated from ferrous iron and salts. The protein was dialyzed against water for 24 hours in the cold, lyophylized, and stored dessicated at 0°C. No detectable iron was found in such preparations when analyzed by atomic absorption spectrophotometry in a Perkin Elmer Model 303 instrument (Perkin Elmer Corp, Norwalk, CT.).

b. Preparation of Apoferritin Subunits

Disaggregation of apoferritin into subunits was accomplished according to the method of Harrison and Gregory (11). A water solution

of apoferritin prepared by heating to 70°C was cooled to 0°C on ice. Two volumes of glacial acetic acid were added and the mixture allowed to stand at 0°C for 1 hr. Dialysis in the cold against 0.037 M glycine-acetic acid buffer, pH 3.0, for 16 hr followed. The extent of subunit formation was determined by subjecting the preparation to analytical, polyacrylamide gel electrophoresis according to the method of Davis (37). Five % gel slabs, 75 x 55 x 3 mm, prepared in the pH 3.0 glycine-acetic acid buffer were cast in a plexiglass mold. Samples were applied by saturating 4 mm x 1 mm strips of Whatman #3 filter paper with up to 5 ul of protein solution and inserting them into slits made in the gel with the sharpened end of a narrow spatula. Electrophoresis was carried out for 60 minutes at 150 volts D.C. and 6-8 milliamp. The protein was stained by immersing the gel in a solution of 0.05 % Ponceau-S in 5 % trichloroacetic acid for 30 min. Background stain was completely washed from the gel by frequent changes of 5 % acetic acid for 72 hrs.

c. Amino Acid Analyses

Quantitative amino acid analyses were made during various stages of this investigation on a Technicon, TSM, Amino Acid Analyzer (Technicon Instruments Corp, Tarrytown, N.Y.). The standard procedure of Spackman, Moore and Stein (38) was followed except that ninhydrin was reduced with hydrazine sulfate instead of stannous chloride. Samples were hydrolyzed in 6.7 N HCl for 16 hrs in evacuated tubes. Norleucine was used as an internal standard. Homoserine lactone was

converted to homoserine prior to analyses by incubating the hydrolystate in 0.1 M pyridine-acetic acid, pH 6.5, for 1 hr at 105°C (39). The solvent was removed in vacuo prior to analysis.

d. Cyanogen Bromide (CNBr) Cleavage of Apoferritin

CNBr cleavage of apoferritin was carried out essentially by the method of Gross and Witkop (33). In early experiments a 240 molar excess over methionine of CNBr (stored dessicated at 0°C) was added to 0.1 micromoles (46.0 mg) of apoferritin dissolved in a minimum of 70 % (v/v) formic acid-water. The mixture was stirred at room temperature for 24 to 72 hr. The digest was diluted 20 fold with distilled water and lyophylized. The product was dissolved in a minimum of 1 M acetic acid, relyophylized and the resulting white, fluffy material stored dessicated at 0°C pending further analysis.

The extent to which cleavage occurred was monitored by subjecting aliquots of the digest to amino acid analysis after hydrolysis in 6.7 N HCl and determining the extent of methionine to homoserine conversion. In later experiments 67 % acetic acid proved to be the solvent of choice.

e. Chromatography on Sephadex Gels

Columns of Sephadex gels were prepared according to standard Pharmacia procedures. Blue Dextran 2000 was used to test column integrity and determine the void volume. Analytical gel chromatography was performed on a 20 x 1.2 cm column of Sephadex G-75 superfine. The eluting solvent was 0.1 M acetic acid at a gravity flow of 0.25 ml/min.

Preparative runs were made on a jacketed 2.5 x 130 cm column of Sephadex G-75. The eluting solvent was 50 % (v/v) formic acid-water and a gravity flow of 1 ml/min was maintained by circulating water at 30°C through the column jacket.

Effluents were passed through the flow cell of a Gilson ultraviolet absorption meter (Gilson Medical Electronics, Middleton, WI) and monitored at 280 nm. Four and 10 ml fractions were collected respectively on a Gilson linear fraction collector LVM1 (Gilson Medical Electronics, Middleton, WI). Aliquots from each tube were analyzed by automated ninhydrin and Sakaguchi (arginine) reactions (14) utilizing Technicon Auto-Analyzer systems (Technicon Instruments, Tarrytown, N.Y.). Figure 1 shows the schematic details of these reactions systems. The contents of tubes containing peptide fractions were concentrated on a Rotary Evapo-mix (Buchler Instruments, New York City, N.Y.), combined diluted 10 fold with distilled water and lyophylized.

The resulting peptide fractions were purified by rechromatography on 2.5 x 42 cm columns of Sephadex G-50 fine or G-25 fine.

f. High Voltage Electrophoresis

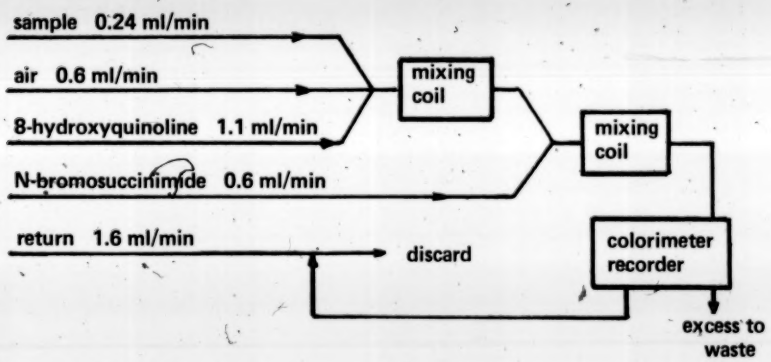
High voltage electrophoresis (HVE) was performed on 250 micron thick layers of cellulose MN-300 spread on 20 x 20 cm glass plates with a mechnical spreader (Reeve Angel, New York City, N.Y.). Volatile buffers used were 1) pH 1.9, acetic acid-formic acid-water (17 :5 :178 v/v); 2) pH 3.5, acetic acid-pyridine-water (10 :1 :189 v/v); 3) pH 4.38, acetic acid-pyridine-water (2 :1 :250 v/v); 4) pH 6.4, acetic

Figure 1. Schematic representation of automated Sakaguchi (arginine) and ninhydrin reactions.

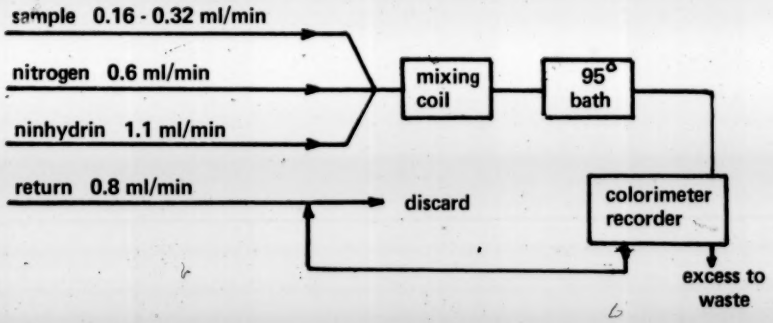
Reagents:

8-hydroxyquinoline: 0.02 % in 3.0 N NaOH
N-bromosuccinimide: 0.1 % in H_2O
Stock ninhydrin: 20 gm ninhydrin and 1.5 gm hydrindantin dissolved in methyl cellosolve - 4 M sodium acetate, pH 5.5 (650: 350 v/v)
Ninhydrin reagent: stock ninhydrin was diluted 1:4 with 50 % methyl cellosolve-water (v/v).

SAKAGUCHI REACTION



NINHYDRIN REACTION



acid-pyridine-water (0.8 :20 :179.2 v/v); 5) pH 8.6, 0.05 M ammonium carbonate.

In general a 5-10 µl sample was placed on the plate and dried in a stream of air. An aerosol of the appropriate buffer was applied by lightly spraying the plate to a matte grey appearance. The wetted plates were placed on the flat aluminum bed of the electrophoresis chamber (Savant model FP-22 flat plate HVE system, Savant Instruments, Hicksville, N.Y.) maintained at 10°C by mechanical cooling. Contact of the layer with the buffer troughs was made through sponge cloth wicks 2 mm thick. A glass plate resting on the wicks served to maintain a buffer saturated atmosphere above the cellulose.

Electrophoresis was conducted for 20-60 min at 1000 volts D.C. and a maximum current of 35 milliamp.

Peptides were visualized by spraying with 0.2 % ninhydrin in acetone by utilizing chlorine and o-tolidine (40) or the Schwarz, Pallansch modification utilizing tert-butyl hypochlorite and starch (41).

Dansylated amino acids separated by HVE were visualized by exposing the plate to long wave (360 nm) ultra violet light in a UV viewing cabinet (Ultra Violet Products Inc., Los Angeles, CA.).

g. End-group Determinations

Analysis of the N-terminal amino acids of the CNBr peptides was made using the dansylation (DNS) procedure of Grey and Hartley (42). The entire procedure was conviently carried out by adapting the Rotary

Evapo-mix to accept 50 x 4 mm glass reaction tubes. In general, 0.1 mg (0.2 n mole - 0.1 μ mole) of unfractionated digest or isolated peptide, dissolved in a minimum of 50 % formic acid-water (v/v), was placed in the reaction tube and evaporated to dryness in vacuo. 20 μl of 0.2 M sodium bicarbonate was added and the mixture again dried in vacuo to remove any traces of ammonia. 20 μl of ammonia free water was added followed by 20 μl of dansyl chloride (DNS-CL) solution (2.5 mg/ml in acetone). Labeling was allowed to proceed for 1 hr at 25°C with gentle swirling. After reaction, the mixture was dried in vacuo and 100 μl of 6.7 N HCl added. The tubes were sealed and hydrolysis carried out for 6-24 hr at 105°C. Removal of the HCl was accomplished in vacuo and the dried hydrolysates were dissolved in 50 % (v/v) pyridine-water prior to examination.

Initial identification of the DNS-amino acids was made by thin layer HVE on cellulose MN-300. Optimum separations were achieved by electrophoresing at 1000 volts D.C. for 60 min in the pH 1.9 buffer and for 30 min in the pH 4.38 buffer.

Identities were confirmed by two-dimensional chromatography on 20 x 20 cm polyamide-6 sheets according to the method of Woods and Wang (43).

The UV fluorescing spots were outlined and tracings of the separations made for future reference.

h. Quantitative Estimation of DNS Amino Acids.

Relative amounts of the N-terminal amino acid in the CNBr peptides were estimated by eluting the HVE separated spots and measuring their fluoresence (44). The fluorescent spots, separated after labeling 0.1 mg of unfractionated peptide digests, were eluted with 2 ml of chloroform-methanol-acetic acid (7 :2 :2 v/v). The fluoresence of each solution was determined in a Turner III fluorimeter (Turner Assoc., Palo Alto, CA.) using Corning 7-60 (350 nm) and Wratten 65A (500 nm) primary and secondary filters. The fluoresence of each eluate, measured in arbitrary units, was compared with those obtained by dansylating pure amino acid standards in a concentration range of 0.001 - 0.1 μ moles which were separated on, and eluted from the same plate as the unknown to correct for possible losses.

i. Electrophoresis of Apoferritin Subunits in Sodium Dodecyl Sulfate Polyacrylamide Gel

Analytical scale disk-gel electrophoresis of dissociated apoferritin subunits was carried out according to the procedure of Weber and Osborne (45), using a Canalco Quick Disc System (Canalco Inc. Rockville, MD). Separations were made in 7.5 % gels containing 0.2 % (w/v) SDS and 0.1 % (v/v) 2-mercaptoethanol at pH 7.0 in 0.1 M sodium phosphate buffer. Electrophoresis was carried out in 6 x 0.6 cm gel tubes at 10 milliamp per tube for three hours. Bromphenol Blue (0.05 % in water) was used as a tracking dye. The gels were stained with Coomassie Brilliant Blue R-250 for 16 hours at room temperature and destained in frequent changes of a solution of 7.5% acetic acid and 5%

methanol in water (v/v) until clear of background. Carbonic anhydrase, carboxypeptidase A, myoglobin and trypsin were used as molecular weight markers.

The mobility of the leading edge of each protein was calculated according to the following formula:

$$\text{Mobility} = \frac{\text{Protein migration (mm)} \times \text{Pre-stained Gel Length (mm)}}{\text{Dye migration (mm)} \times \text{Post-stained Gel Length (mm)}}$$

The mobilities of the marker proteins were calculated for 10 replicate determinations and the mean plotted against their molecular weights expressed on a semi-logarithmetic scale.

Apoferritin was prepared for analyses by dissociating into subunits as previously described. The subunit preparation (0.3 mg/ml) in the pH 3.0 glycine - acetic acid buffer was dialyzed against 500 ml of 0.01 M sodium phosphate buffer, pH 7.0, containing 0.1 % SDS (w/v) and 0.1 % 2-mercaptoethanol for 24 hr at room temperature. This preparation was used for analysis.

The marker proteins were prepared for electrophoresis exactly according to the published method.

j. Total protein was measured by the method of Lowry et. al. (46).

PART III

RESULTS

a. Amino Acid Composition of Horse Spleen Apoferritin

The result of quantitative amino acid analysis of apoferritin is shown in Table II. The number of residues was calculated assuming an average subunit molecular weight of 23,000 and is in excellent agreement with other published studies.

b. CNBr Cleavage of Apoferritin Subunits

First attempts to cleave apoferritin under the conditions described by Gross and Witkop for Ribonuclease (0.1 N HCl, 24 hr at room temperature) were unsuccessful. The choise of acid solvent is governed by the necessity to denature the protein in order to expose the side chains of methionine to attack by the CNBr. Formic acid in concentrations of up to 70 % had been used successfully as a solvent for cleavage of myoglobin (34), β-galactosidase (35) and rabbit immunoglobulin Ig-G (36).

Cleavage was attempted in this solvent as described under methods. Figure 2 shows the best resulting fractionation of the CNBr digest on Sephadex G-75 superfine. The first peak, which accounted for up to 50 % of the digest protein as determined by the Lowry procedure, eluted in the column void volume (Vo) and is believed to be undigested apoferritin oligimer since Sephadex G-75 superfine excludes molecules with molecular weights above approximately 70,000. Five other peaks are clearly distinguished. Although this was an expected result based on

TABLE II

AMINO ACID COMPOSITION OF HORSE SPLEEN APOFERRITIN

RESIDUES/23,000 DALTON SUBUNIT

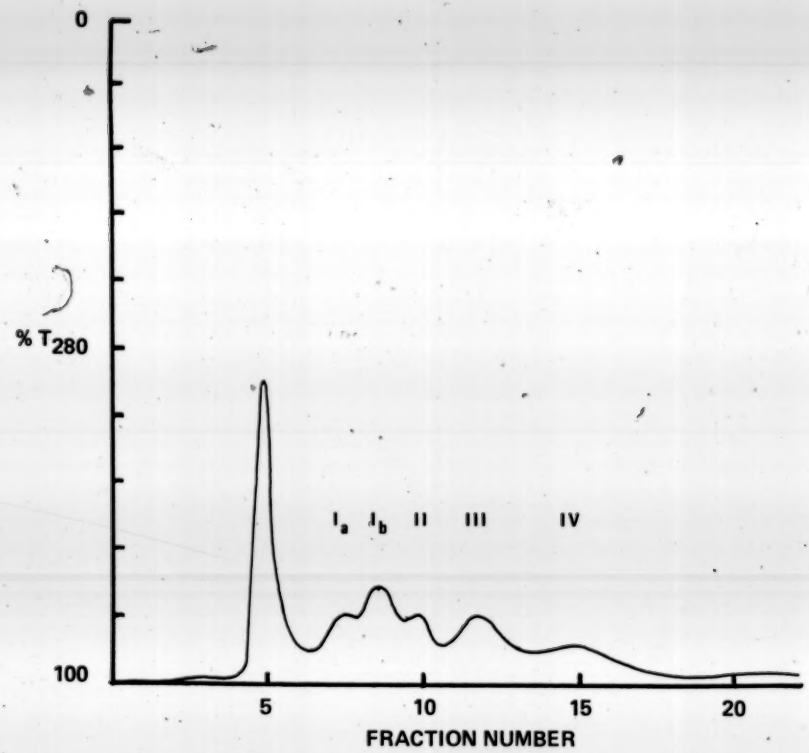
Amino Acid	Residues	Best Integer
Cys	2.42	2
Asp	21.83	22
Thr	7.00	7
Ser	10.68	11
Glu	29.06	29
Pro	2.84	3
Gly	12.50	13
Ala	17.44	17
Val	8.70	9
Met	3.70	4
Ilu	4.29	4
Leu	29.67	30
Tyr	5.71	6
Phe	8.80	9
His	6.58	7
Lys	9.93	10
Arg	11.60	12

Figure 2. Fractionation of the 70 % formic acid - CNBr digest on Sephadex G-75 superfine.

Run parameters

Column: 1.2 x 24 cm
Flow rate: 0.25 ml/min gravity flow
Eluent: 0.1 M acetic acid
Fractions: 4 ml each

Peaks were detected by monitoring the effluent at 280 nm. Other details as described in the text.


0
% T_{280}
100
I_a I_b II III IV
5
10
15
20
FRACTION NUMBER

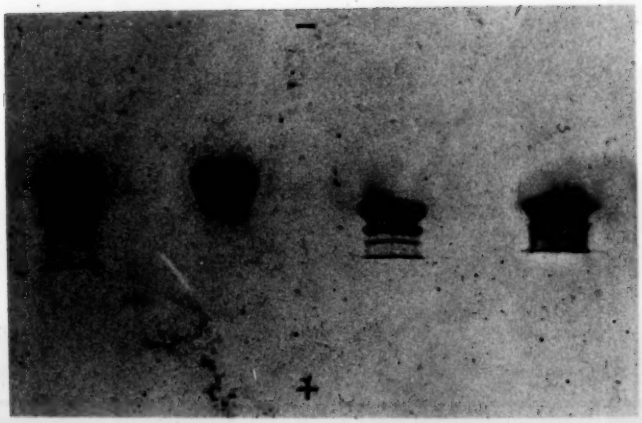
the number of methionine residues, the fact that residual methionine could be detected in rechromatographed fractions of Peaks Ia, Ib and II indicated that cleavage was incomplete.

The report that apoferritin could be dissociated into subunits by incubation in 67 % acetic acid at 0°C (11) presented the possibility that the methionine residues in subunits might be better exposed to attack by CNBr. Disassociation of apoferritin was therefore attempted as described under methods. In order to determine the effectiveness of this procedure, the pH 3.0 dialysate was subjected to analytical polyacrylamide gel electrophoresis in 5 % gel slabs as described under methods. Figure 3 shows in duplicate, a comparison of the electrophoretic patterns pre- and post incubation. Patterns 3 and 4 are identical to those seen previously by Suran and Tarver (14) and represent undissociated apoferritin monomer and higher oligimers. Patterns 1 and 2 show only the faster moving component and indicate that the desired disaggregation could be achieved.

After incubation in 67 % acetic acid at 0°C for 1 hr, a 240 molar excess of CNBr relative to methionine was added and the reaction allowed to proceed for 24 hours at room temperature. Figure 4 shows the Sephadex G-75 superfine fractionation of the resulting digest. The chromatographic pattern was surprising in two respects. The virtual absence of a Vo peak indicated that cleavage had occurred under conditions in which only subunits were present in the reaction mixture. Fraenkel-Conrat (47) had found 67 % acetic to be effective in

Figure 3. Polyacrylamide slab gel electrophoresis of apoferritin oligimers and subunits

Electrophoresis, staining and destaining as described in the text



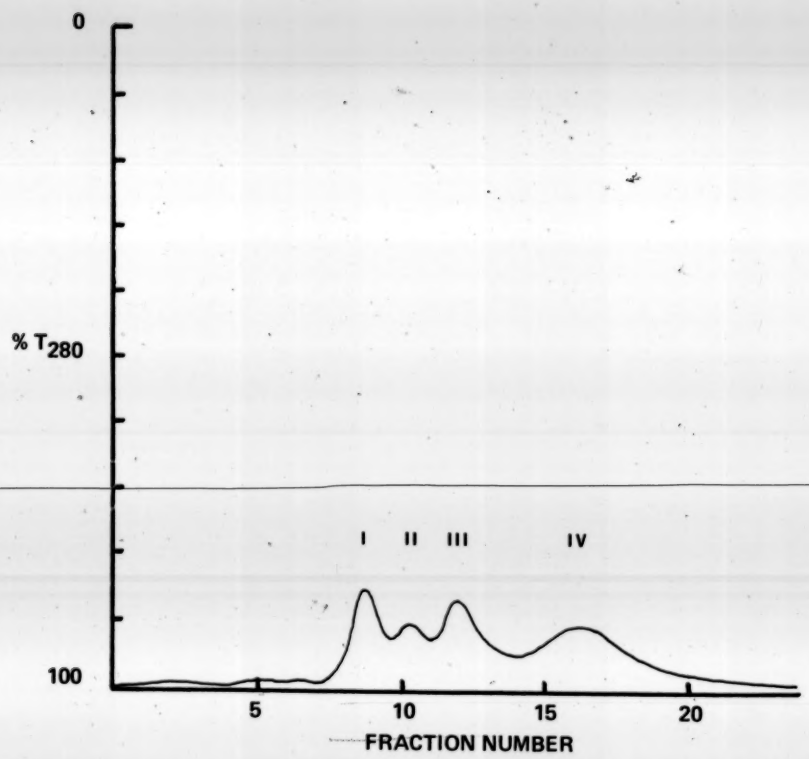
1 2 3 4

Figure 4. Fractionation of the 67 % acetic acid - CNBr digest on Sephadex G-75 superfine

Run parameters

Column: 1.2 x 24 cm
Flow rate: 0.25 ml/min gravity flow
Eluent: 0.1 M acetic acid
Fractions: 4 ml each

Peaks were detected by monitoring the effluent at 280 nm. Other details as described in the text.


0
% T_{280}
100
I
II
III
IV
5
10
15
20
FRACTION NUMBER

dissociating the protein coat of viruses and this type of treatment could prove to be an effective method of exposing available methionine residues in other proteins to attack by CNBr.

More surprising however, was the disappearance of Peak Ia seen in the formic acid digest. Amino acid analysis of the combined Peaks I and II showed a greater than 95 % conversion of methionine to homoserine. Rechromatography of Peaks I, II and III on Sephadex G-50 fine yielded no better resolution. It is possible that Peak Ia seen previously represented either unreacted apoferritin monomer or a smaller, unfragmented peptide. The possibility that there were two peptides of virtually the same size and therefore eluting in identical volumes could not be overlooked. High voltage electrophoresis at pH 1.9 of the CNBr digest yielded only a maximum of four spots when visualized with the chlorine-o-tolidine spray but smearing tended to obscure resolution and the results were inconclusive.

Fractionation of the CNBr digest on a long column of Sephadex G-75 is shown in Figure 5. Peak IV contained no detectable arginine as determined by the automated Sakaguchi reaction.

c. End Group Analysis of CNBr Peptides by Dansylation

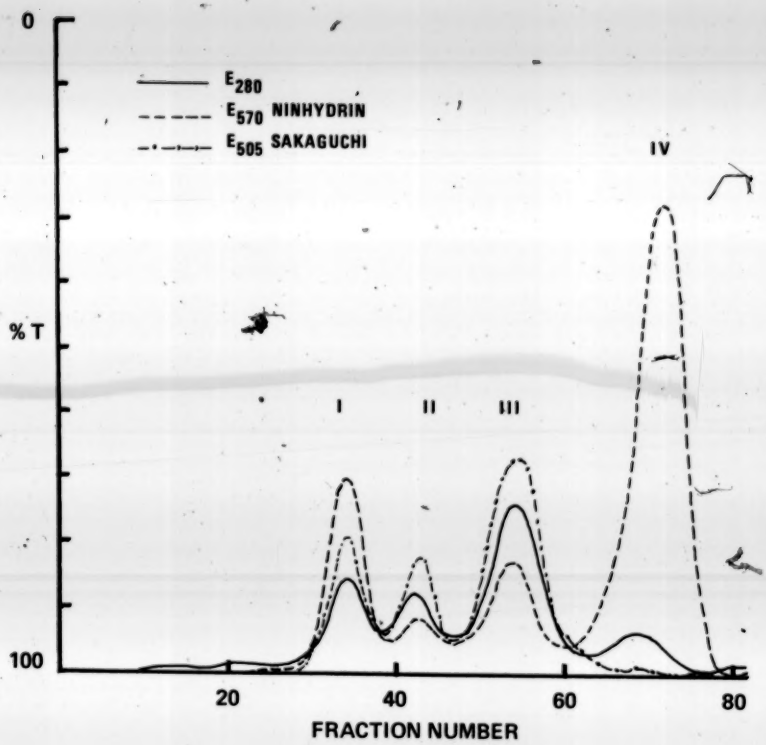
The dansylation procedure of Grey and Hartley (42) had been applied to the N-terminal analysis and sequence determination in an increasing number of peptide and protein investigations (48)(49)(50) and because of its ease of application could provide a rapid estimation of the number of peptides in the CNBr digest. Only three dansyl

Figure 5. Preparative fractionation of the 67 % acetic acid - CNBr digest on Sephadex G-75.

Run parameters

Column: 2.5 x 130 cm
Temperature: 30°C
Flow rate: 1 ml/min gravity flow
Eluent: 50 % formic acid-water (v/v)
Fractions: 10 ml each

Peaks were detected by monitoring the effluent at 280 nm and by the automated Sakaguchi (arginine) and ninhydrin reactions. Details as described in the text.


0
100
% T
E_{280}
E_{570} NINHYDRIN
E_{505} SAKAGUCHI
I
II
III
IV
20
40
60
80
FRACTION NUMBER

derivatives were detected in the digest after preparing the derivatives and analysis by HVE as described under methods. These were tentatively identified as glutamic acid, glycine and isoleucine. Two dimensional chromatography on polyamide-6 sheets confirmed their identity. An aliquot of the CNBr digest labeled and hydrolyzed for 16 hr gave excellent yields of DNS-Glu and DNS-Gly. A 24 hr hydrolysis time was necessary to achieve good yields of DNS-Ilu due to its apparent resistance to hydrolysis (51). Figure 6 shows a tracing of the two dimensional separation.

No conclusion can be made as to whether N-terminal glutamine was present in the CNBr digest since it would be converted to DNS-Glu during the procedure.

Since HVE of the unfractionated CNBr digest was inconclusive in separating the peptides in the unfractionated digest and the possibility of two peptides with the same N-terminal acid could not be overlooked an attempt was made to quantitate the relative amounts of the labeled terminal residues by eluting the spots separated by HVE and measuring their fluoresence. Separations made on polyamide-6 sheets could not be used for this purpose due to non-reproducable background blanks.

Table III shows the resulting amounts of fluoresence expressed in arbitrary units after correcting for background and possible losses as described under methods.

Application of this technique, although crude, indicated

Figure 6. Identification of the N-terminal amino acids in the CNBr digest.

Dansylation of the digest was carried out as described in the text. Separation of the dansyl derivatives on a 20 x 20 cm sheet of polyamide-6 was carried out by chromatographing first in formic-acetic acid (200:3 v/v), air drying, and then in a direction 90^{o} to the first in benzene-acetic acid (9:1 v/v). Pure standards of the DNS-amino acids in question were co-chromatographed in each direction on the edges of the sheet. Visualization was made by exposing the sheet to long wave ultra-violet light. Dansylamide and dansylsulfonic acid fluoresce orange and light blue respectively.

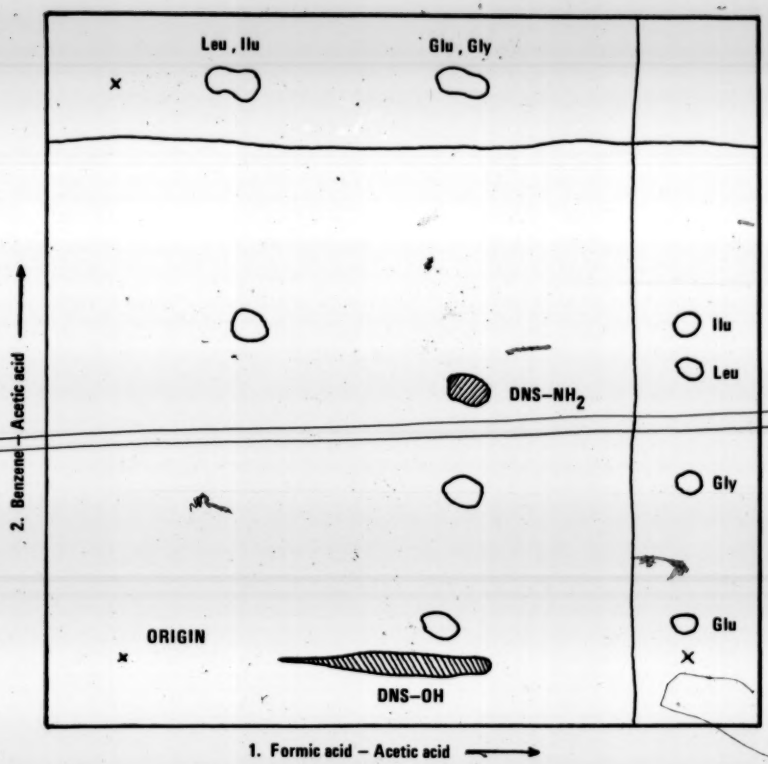

Leu , Ilu
Glu , Gly
Ilu
Leu
DNS–NH2
Gly
Glu
ORIGIN
DNS–OH
2. Benzene – Acetic acid
1. Formic acid – Acetic acid

TABLE III

FLUORESENCE OF N-TERMINAL DNS AMINO ACIDS

DNS - Amino Acid	Standard (0.001 μ mole)	CNBr Digest
Glu	7.62	0.46
Gly	10.43	0.53
Ilu	9.38	0.51

Separation of DNS - amino acids by HVE on cellulose MN-300. Fluoresence measured in arbitrary units after eluting the spots from the thin layer plate. Details as in text.

that the separated N-terminal amino acids were present in essentially equimolar amounts and therefore only three dansylable peptides were present in the CNBr digest.

This result leads to the conclusion that three methionine residues are present in the apoferritin subunit.

d. Estimation of the Apoferritin Subunit Molecular Weight by SDS-Polyacrylamide Gel Electrophoresis.

The presence of three methionine residues cast doubt on the previously determined number of each amino acid in the subunit. The number of amino acid residues had been calculated on the basis of a 23,000 Dalton molecular weight. If this value of the subunit molecular weight was incorrectly high this would account for the apparent over estimation of four methionine residues. Further indication that the actual subunit molecular weight might be lower than previously thought came from an independent laboratory.[1]

Weber and Osborne had previously shown that protein molecular weights could be reliably estimated within ±10 % by SDS-polyacrylamide gel electrophoresis (45). Hofmann and Harrison (9) had shown that dissociation of apoferritin into subunits required SDS-protein ratios higher than 1:3 (w/w). It was therefore decided to dissociate the monomer with 67 % acetic acid prior to electrophoresis. When the dissociated subunits of apoferritin were examined in 7.5 % gels as described under methods a mobility value of 0.662 ± 0.010

1. Personal communication from R. R. Crichton.

was obtained for 10 replicate determinations. The average mobilities were obtained for 10 replicate runs of the marker proteins under identical electrophoretic conditions and a standard curve was obtained by plotting these values against the logarithm of their molecular weights.

Figure 7 shows the results of the analytical polyacrylamide gel electrophoresis.

Figure 8 shows the resulting standard plot of the marker protein mobilities versus the logarithm of their molecular weights. Extrapolation of the determined apoferritin mobility leads to a molecular weight of 18,700 ± 600.

e. Characterization of Peptide IV

Peak IV from the Sephadex G-75 fractionation was rechromatographed on Sephadex G-50 fine. The resulting slowest eluting fraction was collected, lyophylized and further purified by chromatography on Sephadex G-25 fine. The resulting homogeneous peak which gave a negative reaction for arginine by the automated Sakaguchi procedure showed low absorbance at 280 nm and was located in the column effluent by the automated ninhydrin method. After collection and lyophylization the peptide was subjected to determinations for amino acid composition and N-terminal residue as described under methods.

Table IV shows the results of quantitative amino acid analysis expressed as micromoles per milligram of peptide and relative ratios.

The N-terminal amino acid residue was determined by the

Figure 7. SDS-polyacrylamide gel electrophoresis of apoferritin subunits and marker proteins.

The protein bands shown are as follows:

(1) From top to bottom; carboxypeptidase A (34,600); carbonic anhydrase (29,000); trypsin (23,300); myoglobin (17,200).
(2) Dissociated apoferritin
(3) Trypsin (23,000); dissociated apoferritin
(4) Myoglobin (17,200); dissociated apoferritin

Molecular weights from ref. (45). Details of electrophoresis as described in the text.

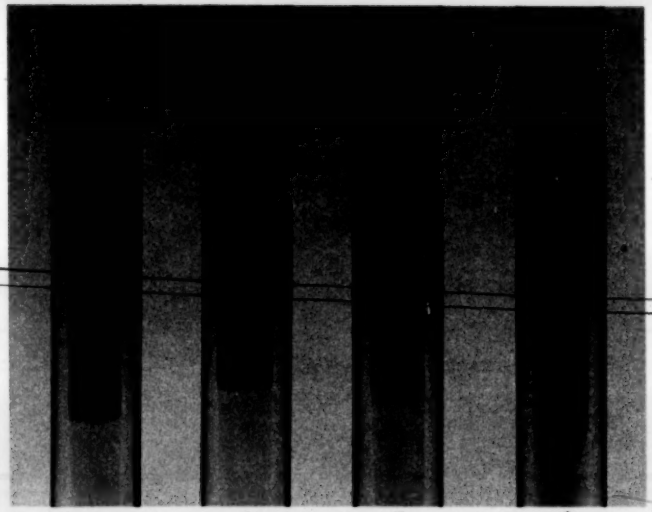

1
2
3
4

Figure 8. Molecular weight estimation of apoferritin subunits

The mobilities of the marker proteins are the means of 10 replicate determinations. The mobility of dissociated apoferritin is indicated by the broken line and is the mean of 10 replicate determinations.
Molecular weights of marker proteins from ref. (45).
Details as described in the text.

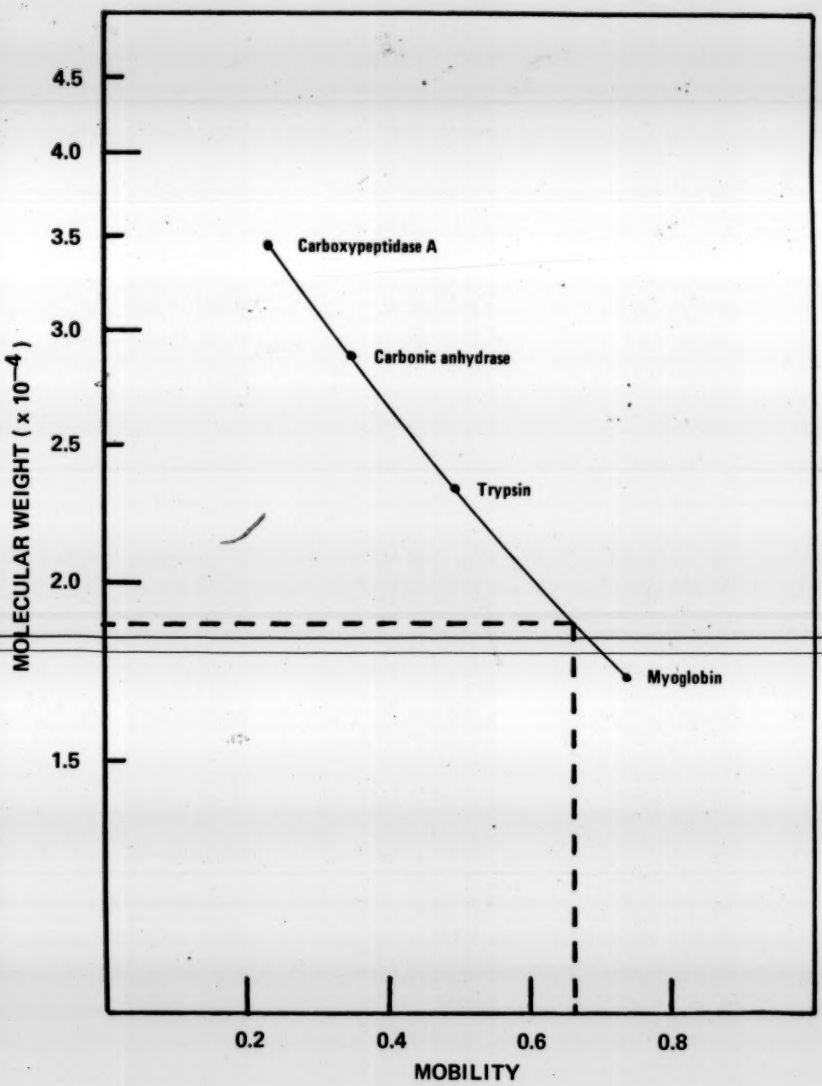

4.5
4.0
3.5
3.0
2.5
2.0
1.5
MOLECULAR WEIGHT (x 10⁻⁴)
Carboxypeptidase A
Carbonic anhydrase
Trypsin
Myoglobin
0.2
0.4
0.6
0.8
MOBILITY

TABLE IV

AMINO ACID COMPOSITION OF CNBr PEPTIDE IV

Amino Acid	μ moles/mg	Residues
Asx[1]	1.10	1
Glx[2]	0.89	1
Gly	0.97	1
Leu	1.06	1
Phe	0.80	1
Ser	0.99	1
H-Ser	1.02	1

1. Asx: Aspartic acid or asparagine

2. Glx: Glutamic acid or glutamine

dansylation procedure and identified by two dimensional chromatography on polyamide-6 sheets as Glutamic Acid.

The peptide was subjected to HVE at pH 1.9, pH 3.5, pH 6.3 and pH 8.6 as described under methods. Figure 9 shows a composite tracing of the electrophoretic patterns. The neutral behavior at pH 3.5 and pH 6.3 indicates blocking of both the γ carboxyl group of glutamic acid and the β carboxyl group of aspartic acid. The behavior at pH 8.6 however is anomolous in that the presence of free γ and β carboxyl groups is apparent.

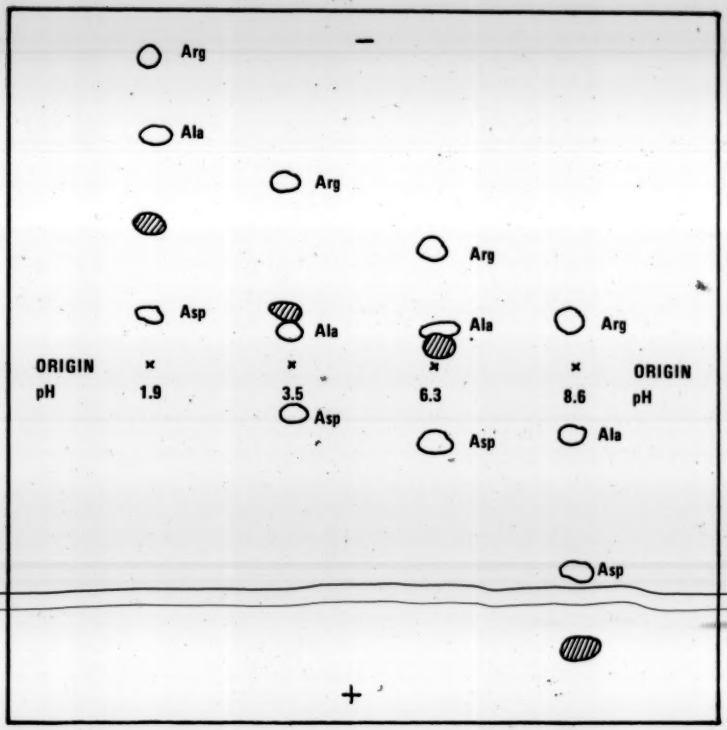
The possibility that deamidation and spontaneous cyclization of an N-terminal glutamine residue to pyrrolidonecarboxylic acid had taken place during CNBr cleavage and subsequent peptide isolation can not be overlooked. However, such a cyclic derivative would not react with DNS-CL. Coupling with DNS-CL did take place discounting such a cyclization.

Deamidation could have occurred under the conditions of HVE at pH 8.6.

No definite conclusions can be drawn from the electrophoretic behavior of peptide IV.

Figure 9. Electrophoretic behavior of peptide IV.

Composite tracing of electrophoretic patterns in four buffer systems. All separations on cellulose MN-300. Visualization by ninhydrin spray. Details as described in the text.


−
Arg
Ala
Arg
Arg
Asp
Ala
Ala
Arg
ORIGIN
pH
1.9
3.5
6.3
8.6
ORIGIN
pH
Asp
Asp
Ala
Asp
+

PART IV

DISCUSSION

Previous estimates of the number of subunits and their molecular weights were based on the following physical and chemical data:

1. Native apoferritin was determined to have a molecular weight ranging from 460,000 - 480,000 on the basis of x-ray diffraction, crystal density and light scattering analyses (15)(52). Investigations utilizing sedimentation diffusion and approach to equilibrium methods demonstrated that the molecular weights of the dissociated subunit ranged from 25,000 to 27,000 (15).

2. Twenty-one tryptophan residues were found in the apoferritin oligimer (16) and the number of peptides released from tryptic digests seemed consistent with the arginine and lysine content (17). Carboxypeptidase B released nearly 20 moles of arginine and 19.6 moles of bound acetyl groups were found per mole of apoferritin (19). The physical and chemical data were consistent with the model proposed by Harrison (6) in which 20 subunits were arranged at the vertices of a pentagonal dodecahedron.

The data presented in this study is in disagreement with this model. This conflict can be resolved however by consideration of the following points:

a. The previously published values for the molecular weight of horse spleen apoferritin were derived from sedimentation equilibrium studies using the partial specific volume of 0.747 calculated by

Rothen (5). The classically prepared apoferritin used in these studies was later shown by Suran and Tarver (14) to be a heterogenous mixture containing a main component sedimenting at 17.6 S, and minor components of 25 S and 34 S. The presence of diners, trimers and possibly higher oligimers can be expected to affect the pycnometric determination of the partial specific volume and therefore lead to an erronous estimate of molecular weight. Furthermore, the tendancy to form aggregates displayed by apoferritin (11) could also be expected to adversely affect molecular weight determinations. Bjork and Fish (13) redetermined the molecular weight of native apoferritin by meniscus depletion sedimentation equilibrium experiments under a variety of solution conditions and arrived at values ranging from 440,000 to 460,000, substantially lower than those reported earlier.

b. The subunit molecular weight of 25,000 to 27,000 determined in sedimentation equilibrium studies after dissociation with sodium dodecyl sulfate was based on the amount of the detergent bound after equilibrium dialysis. Critical to such a study is the accurate determination of the amount of SDS bound. Reynolds and Tanford (53) have determined a ratio of 1.4 gm of SDS per gram of globular protein. This is in substantial disagreement with the data of Hofmann and Harrison (9) who found that 0.5 gm of SDS was bound per gram of apoferritin. Failure to completely remove all of the SDS would lead to an underestimation of the amount bound to the protein and thus to an overestimation of the subunit molecular weight.

c. Molecular weight estimates by chemical methods can also give rise to error. One of the difficulties in using the amino acid composition of the subunit to estimate its minimum molecular weight is that those residues which are present in relatively small amounts are the most difficult to accurately determine. The colorimetric method used by Harrison et. al. (16) to determine tryptophan is quite complex and the possibility of error cannot be overlooked. Likewise the method used to determine the amount of acetic acid released from the serine-bound acetyl groups (54) has been shown to give values which are lower than actual (55). The same can be said for estimates based on the amount of C-terminal arginine released after treatment with carboxypeptidase B. Estimates based on peptide maps may be inaccurate due to non-specific or incomplete digestion.

The data from the present study leads to the conclusion that the subunit molecular weight of apoferritin is substantially lower than previously thought. Recalculating the amino acid composition on the basis of this lower molecular weight results in a value of three methionine residues per subunit as shown in Table V. The fact that only three peptides are found in the CNBr digest, which are capable of reacting with DNS-CL, is consistent with the presence of three methionine residues since the fourth peptide is known to have N-acetyl serine as its N-terminal residue and is therefore unreactive.

A molecular weight of 18,700 ± 600 would give a value of 374,000 ± 12,000 for the weight of undissociated apoferritin based on

TABLE V

AMINO ACID COMPOSITION OF HORSE SPLEEN APOFERRITIN

RESIDUES/18,700 DALTON SUBUNIT

Amino Acid	This Study	Ref (a)	Ref (b)	Best Integer
Cys	1.97	1.87	2.81	2
Asp	17.75	17.10	17.11	17
Thr	5.69	5.45	5.43	5
Ser	8.68	9.39	8.87	9
Glu	23.62	23.73	23.58	24
Pro	2.30	2.04	2.79	2/3
Gly	10.16	9.69	9.76	10
Ala	14.18	14.03	13.81	14
Val	7.07	6.98	6.98	7
Met	3.01	2.92	2.83	3
Ilu	3.49	3.71	3.46	3/4
Leu	24.12	24.81	24.67	24
Tyr	4.64	5.06	4.93	5
Phe	7.16	7.21	7.23	7
His	5.35	5.80	5.71	6
Lys	8.08	8.86	8.64	9
Arg	9.43	9.74	9.34	10

(a) Williams, M.A. and P.M. Harrison. Biochem. J. 110 265-280 (1968).

(b) Crichton, R.R. Biochim. Biophys. Acta. 194 34-42 (1969).

a 20 subunit model and agrees poorly with the previously determined range of 460,000 to 480,000. A 24 subunit model would have a calculated weight of 448,000 ± 14,000. This value is in good agreement with the range of 440,000 to 460,000 reported by Bjork and Fish (13).

A model consisting of a 24 subunits situated at the vertices of a snub cube was originally proposed by Harrison in 1959 (8).

PART V

ADDENDUM

Since the completion of the work described here the following corroborating reports have appeared in the literature.

1. Sedimentation equilibrium determinations using UV optics have enabled measurements to be made at protein concentrations which preclude aggregation or other non-ideal behavior. Applying this technique to study apoferritin, Crichton et. al. obtained a value of 443,000 for the molecular weight of the oligomer (56).

2. The subunit molecular weight of apoferritin has been estimated by gel filtration in 6 M guanidinium chloride (57) and by sedimentation equilibrium analysis in 0.01 M glycine-HCl buffer after dissociation in 67 % acetic acid. Molecular weights of 18,500 ± 500 were found in each case.

3. A preliminary report that CNBr cleavage of undissociated apoferritin released four peptides has been made (58). The N-terminal amino acids determined by a different method are in agreement with those reported here.

4. The quarternary structure of apoferritin has been reassessed (59). Comparison of the three dimensional function of cubic apoferritin with similar functions calculated for model structures of 20 and 24 subunits leads to the conclusion that the apoferritin molecule consists of 24 subunits arranged in octahedral symmetry.

PART VI

SUMMARY

Ferritin was isolated from horse spleen. The protein moiety, apoferritin, prepared by reduction and removal of the iron micelle, was subjected to cleavage by cyanogen bromide as a first step in structural studies to determine the number of subunits in the apoferritin monomer and to ascertain their equivalence.

Four peptides were found in the cyanogen bromide digest, three of which have N-terminal glutamic acid (or glutamine), glycine and isoleucine respectively. One of the peptides has been isolated by Sephadex gel chromatography and found to have the composition: aspartic acid (or asparagine), glutamic acid (or glutamine), glycine, leucine; phenylalanine, serine, homoserine.

The molecular weight of the dissociated apoferritin was determined by sodium dodecyl sulfate - polyacrylamide gel electrophoresis and found to be 18,700 ± 600.

The data suggest that the subunit molecular weight is lower than previously thought and that apoferritin may consist of 24 subunits.

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